SUBSTITUTE SPECIFICATION

EPIMEREDINOSIDE A, ORAL PHARMACEUTICS CONTAINING THE SAME, AND PREPARATORY AND DETERMINATION METHODS

BACKGROUND OF THE INVENTION

1. Field of the Invention

[0001]

This invention relates to the field of TCM pharmaceutics, mainly dealing with anti- female menopausal-syndrome-effective epimeredinoside A, and epimeredinoside A -contained pharmaceutics of *Epimeredi indica* extract and the pharmaceutics' preparatory method.

2. Background of the Related Art

[0002]

Estrogen and its pharmaceuticss have been applied widely for the treatment of menopausal syndrome for a long time. However, it is hard to gain acceptance by women due to its many side effects and adverse reaction, even leading to cancer. Therefore, there is no satisfactory clinical drug at present.

[0003]

Epimeredi indica (L.) Rothmalex, Guang-Fang-Feng, also named Fang-Feng-Cao, is recorded in The Dictionary of Traditional Medicine and is the whole plant of *Epimeredi indica* in the Labiatae family. It has been used in the treatment of many disorders such as cold with fever, disgorging, abdominal pain, bones and muscles pain, pyocutaneous disease, eczema, hemorrhoids and so on. It

is used in the formula of Guanfang Ganmao Pills recorded in Volume 20 of *Zhong-Yao-Cheng-Fang-Zhi-Ji* (the TCM Pharmaceutics of Patent Formula) published by the Ministry of Public Health of the People's Republic of China, .

[0004]

A new use for *Epimeredi indica* root has been announced in Chinese Patent No.02110522.7 by the inventor. *Epimeredi indica* root has the effects of ameliorating ovary function and regulating estrogen and progestogen, therefore it can be used to prepare drugs and health care products to treat and prevent many diseases due to the imbalance of estrogen and progestrogen.

SUMMARY OF THE INVENTION

[0005]

The present invention further develops pharmaceutics of *Epimeredi indica* root extract on the basis of Chinese Patent No.02110522.7, about a novel oral pharmaceutics with clear active constituent and its content and stable quality.

[0006]

The present invention announces all kinds of pharmaceutics related to any oral pharmaceutics composed of *Epimeredi indica* root extract and pharmaceutical adjuvant. This extract is obtained from extracts of *Epimeredi indica* root, after being extracted by water and concentrated by distillation, containing 0.10% to 1.50% of epimeredinoside A.

[0007]

Pharmaceutical adjuvants involved in the present invention are all common

adjuvants in regular pharmaceutics. The oral pharmaceutics are any oral dosage forms widely used in the medical area including hard capsule, soft capsule, granule, tablet, oral liquid and so on.

[0008]

Another technical point announced in the present invention is the preparatory method of the extract and determination method of active constituents in it.

[0009]

A preparatory method for *Epimeredi indica* root extracts of the present invention comprises the following steps:

1. The roots of *Epimeredi indicia* were powdered. Then, a 10 times amount of water was added, and extraction conducted two times, for 1~2 hours per time. After filtration, it was concentrated as *extracta sicca* to a density of 1.01 to 1.08(25~30℃), then dried by spray or vacuum. The content of epimeredinoside A in this extract was 0.10 to 1.50% as determined by HPLC.

2. Proportions of extracts and adjuvants were mixed well to prepare various pharmaceutics conventionally by wet or dry granulation.

[0010]

The content determination method of Epimeredinoside A in extracts of *Epimeredi indica* root of the present invention comprises the following steps of:

[0011]

1. Apparatus and Materials:

Instrument: Agilent 1100 HPLC system

Standard: epimeredinoside A

Chemical reagents: methanol，acetonitrile，distilled water and other reagents were HPLC grade

Sample: Extracts of *Epimeredi indica* root (Shanghai Yaogang Biotechnology Ltd.Co.)

[0012]

2. Chromatographic conditions:

Chromatographic column: Discovery C_{18} (250mm ×4.6 mm, 5μm)

Mobile phase: acetonitrile:water = 27:73

Flow rate: 1.0ml/min

Column temperature: room temperature

Detection wavelength: 320nm

Injection volume: 20ul

[0013]

3. Calibration curve:

[0014]

□ Preparation of standard stock solutions: The standard was prepared by weighing 4.95 mg, and dissolving and diluting with methanol in a 25 ml volumetric flask to obtain standard stock solutions for the calibration curves.

[0015]

□The Calibration Curves: From the stock solution, 0.4, 0.8, 1.2, 1.6, and 2.0 ml were weighed, respectively, dissolved, and diluted with methanol in 2 ml

volumetric flasks to obtain standard solutions at concentrations of 39.6 μg/ml, 79.2 μg/ml, 118.8 μg/ml, 158.4 μg/ml, and 198 μg/ml, respectively.

[0016]

A total of 20 μL of each standard solution was subjected to HPLC quantitative analysis. A calibration curve was generated to confirm the linear relationship between the peak area ratio (Y axis) and the concentrations of the standard (X axis) in the test samples. The calibration curves were found to be linear and could be described by the regression equations Y=20.139 X – 154.35, with coefficient R^2 = 0.9994. The range of calibration curves was 0.792 – 3.96 μg, and the retention time of epimeredinoside A was 9.55 min.

[0017]

4. Sample determination

[0018]

Preparation of the standard solutions: The standard was accurately weighed, and dissolved and diluted with methanol in a volumetric flask to obtain standard solutions. A total of 20 μL of standard solution was subject to HPLC quantitative analysis and the peak area was recorded. The content of epimeredinoside A was calculated using the calibration curves accordingly, see Fig 2.

[0019]

Preparation of the sample solutions: The extracts of *Epimeredi indica* root (176.66 mg) were accurately weighted, and extracted by ultrasonication at room temperature for 2 times, then centrifuged. The supernatants were combined and diluted with water in a 10 ml volumetric flask. The solution was filtered through a

syringe filter (0.45 μm).

[0020]

The sample solutions were subjected to HPLC analysis as described above. The content of epimeredinoside A in the samples was calculated according to the calibration curves.

[0021]

Formula for calculation is as follows:

Y=20.139X-154.35

Y : value of peak area

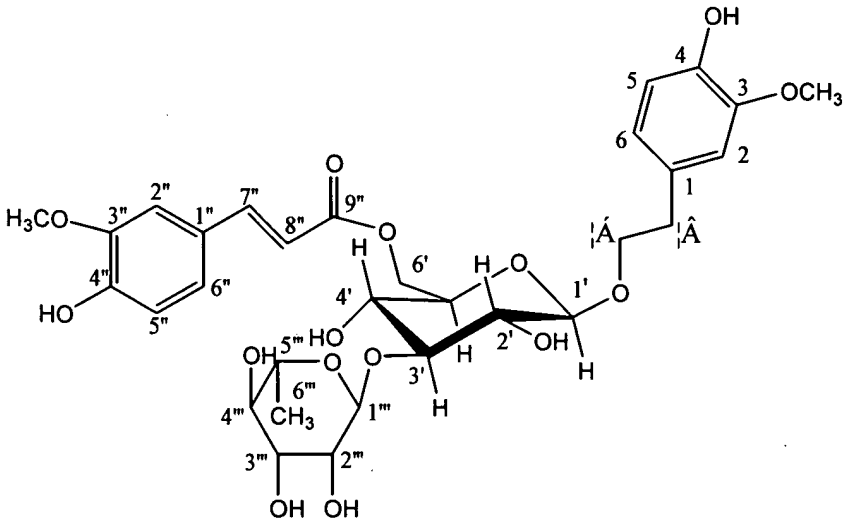
X: value of sample concentration (μg/ml)

[0022]

The content of epimeredinoside A in a sample is demonstrated as X*10/*amount of sample*100%.

[0023]

The Epimeredinoside A used in the present invention is an active compound obtained from extracts of *Epimeredi indica* root through isolation and purification. Extracts of *Epimeredi indica* root were extracted with n-butanol. The soluble extracts were then chromatographed on macroporous resin and a C-18 silicon column, eluted with ethanol gradient, collected and assayed by TLC. The ethanol elute was concentrated to obtain epimeredinoside A. Figure 2 is its chromatogram of HPLC. Its structure is showed as follows:



[0024]

Validation of the HPLC methods for determination epimeredinoside A in the present invention:

[0025]

(1) Calibration curve:

[0026]

□ Preparation of standard stock solutions: The standard was prepared by weighing 4.95 mg, and dissolving and diluting with methanol in a 25 ml volumetric flask to obtain standard stock solutions for the calibration curves.

[0027]

□The Calibration Curves: From the stock solutions, 0.4, 0.8, 1.2, 1.6, and 2.0 ml were weighed, respectively, dissolved, and diluted with methanol in 2 ml volumetric flasks to obtain standard solutions at concentrations of 39.6 μg/ml, 79.2 μg/ml, 118.8 μg/ml, 158.4 μg/ml, and 198 μg/ml, respectively.

[0028]

A total of 20 μL of each standard solution was subjected to HPLC quantitative analysis. A calibration curve was generated to confirm the linear relationship between the peak area ratio (Y axis) and the concentrations of the standard (X axis) in the test samples. The calibration curves were found to be linear and could be described by the regression equations Y=20.139 X – 154.35, with coefficient R^2 = 0.9994. The range of calibration curves was 0.792 – 3.96 μg, and the retention time of epimeredinoside A was 9.55 min.

[0029]

Peak area

Number	1	2	3	4	5
Sample concentration(μg/ml)	39.6	79.2	118.8	154.4	198
Peak area (mAU)	612.811	1472.17	2234.391	3036.277	3802.776

[0030]

Calibration of epimeredinoside A is given in figure 1.

[0031]

(2) Precision

[0032]

To imbibe a standard solution at a concentration of 0.198mg/ml for precision study under the above HPLC chromatographic conditions, then inject the above standard solution six times consecutively.

[0033]

Number	Peak area	X	RSD (%)

1	3802.776	3815.223	0.824
2	3806.568		
3	3879.024		
4	3796.254		
5	3802.456		
6	3804.259		

[0034]

The results showed that the precision of this method is preferable.

[0035]

(3) Stability

[0036]

Peak area of standard solution was assayed at 0, 4, 8,12h with an injection volume of 20ul per time.

Number	1	2	3	4
Peak area	3785.21	3749.56	3802.54	3855.23
Mean	3798.135			
RSD (%)	1.16			

[0037]

(4) Reproducibility

[0038]

Five samples that have the same batch number were prepared for measurement according to the criteria of the sample assay procedure mentioned above.

[0039]

Peak area of epimeredinoside A in a sample solution was assayed with an injection volume of 20μl.

Number	1	2	3	4	5
Peak area	522.824	531.245	536.258	522.356	514.252
Mean	525.387				
RSD (%)	1.63				

[0040]

(5) Recovery

[0041]

The determined samples were weighed accurately and the standard epimeredinoside A solutions were added into the samples accordingly, and the content of epimeredinoside A in samples were determined under the same conditions as described above.

[0042]

NO.	Sample/μg	Added/μg	Analysis/μg	Recovery	Average	RSD(%)
1	38.643	31.68	68.495	97.400		
2	38.643	31.68	66.455	94.500		
3	38.643	39.6	72.922	93.199	98.292	5.26
4	38.643	39.6	74.8	95.600		
5	38.643	47.52	99.362	102.552		
6	38.643	47.52	91.764	106.500		

[0043]

The results showed that a sensitive and stable analysis method for the determination of *Epimeredi indica* Root Extract was established.

[0044]

The inventive pharmaceutics of *Epimeredi indica* Root Extract do not contain any hormone. No progesterone is needed to be taken to prevent a side effect after using the drug. It is compatible for a female in menopause that the drug has doubtless effect in clinic, stability, controllable and safety. Furthermore, a new approach was provided for patients which need to use estrogen but with contraindication of hormone.

BRIEF DESCRIPTION OF THE VARIOUS VIEWS OF THE DRAWING

[0045]

Fig.1: Calibration curve of the epimeredinoside A;

Fig.2: HPLC chromatogram of epimeredinoside A; and

Fig.3: HPLC chromatogram of Epimeredi indica Root Extract.

DETAILED DESCRIPTION OF THE INVENTION

[0046]

Example 1 - Preparation of epimeredinoside A

[0047]

(1) The dried and powdered root of *Epimeredi indica* was extracted with 10 fold water for 2 hours, and filtered. The residue was extracted with 8 fold water for 2 hours again, and filtered. The filtrates were combined and evaporated under vacuum to afford *Epimeredi indica* Root Extracts.

[0048]

(2) The 6 kg of *Epimeredi indica* Root Extracts was extracted with 10 fold water for 3 times, and the solvent was evaporated to 600 ml. The residues were extracted with aqua-saturated n-butanol for 3 times (400 ml/ time). The n-butanol solvent was evaporated under vacuum. The extracts of n-butanol were dissolved in water and chromatographed in a macroporous resin column (AB-8, Nankai Chemistry Factory, Tianjin). The chromatographic column was eluted with gradient mixtures of 20%, 50% and 95% aqueous ethanol successively. The elutes of 50% ethanol were concentrated and then dissolved with 50% aqueous methanol. The

samples of 50% methanol were chromatographed on a RP-C18 silica column, eluted with 50% aqueous methanol to produce epimeredinoside A.

[0049]

The structure of epimeredinoside A was elucidated by UV, IR, ESI, HRESI, NMR, 2D-NMR (COSY, HMQC, HMBC, NOESY) data. Epimeredinoside A, mp 139~142□, molecular formula of $C_{13}H_{40}O_{15}$ and the molecular weight 652, was isolated. The ^{1}H NMR (500MHz) and ^{13}C NMR (125MHz) spectral data of Epimeredinoside A ($CDCl_3$) was shown in Table 1.

[0050]

Table 1: ^{1}H NMR (500MHz) and ^{13}C NMR (125MHz) spectral data of Epimeredinoside A ($CDCl_3$)

Ferulic acid	δC	ΔH	Aglycone	ΔC	δH
1	127.68		1	132.69	
2	111.66	7.15 (d,2)	2	117.00	6.69 (d,2)
3	150.64		3	147.47	
4	149.36		4	147.33	
5	116.47	6.80 (d,8)	5	112.81	6.65 (d,8)
6	124.27	7.02 (dd,8,2)	6	121.11	6.61 (dd,8,2)
7	147.10	7.62 (d, 16)	α	36.71	2.80 (t,7)
8	115.28	6.39 (d,16)	β	72.31	3.5 –4.2
9	169.07		OCH3	55.40	3.76 (s)

OCH3	55.44	3.86 (s)			
Glucose	**δC**	**ΔH**	**Rhamnose**	**ΔC**	**δH**
1	104.39	4.33 (d,8)	1	102.73	5.18 (d,1)
2	75.66	3.5—4.2	2	72.34	3.5 –4.2
3	84.08	3.53 (m)	3	72.25	3.5 –4.2
4	70.54	3.5 – 4.2	4	73.99	3.5 –4.2
5	75.37	3.5 –4.2	5	70.05	3.5 –4.2
6	64.48	4.41 (m)	6	17.88	1.25 (d,6)

[0051]

Example 2 - Preparation and Quantitative Analysis of *Epimeredi indica* Root Extract

[0052]

A: The dried and powdered root of *Epimeredi indica* was extracted with 10 fold water, and filtered. The residue was extracted with 8 fold water for 2 hours again, and filtered. The filtrates were combined and concentrated under vacuum to obtain the extracts of *Epimeredi indica* Root.

[0053]

B: Quantitative Analysis

[0054]

1. Apparatus and Materials

Apparatus: Angilent 1100 HPLC system.

Standard: Epimeredinoside A

Chemical reagents: Methanol, acetonitrile, water and other

chemical reagents were HPLC-grade.

Samples: Extracts of *Epimeredi indica* Root (Shanghai Yaogang Biotech Co. Ltd)

[0055]

2. Chromatographic conditions

Column: Discovery C18 (250mm x 4.6 mm, 5μm)

Mobile phase: Acetonitrile : Water = 27: 73

Flow rate: 1.0 ml/min

Column temperature: Room temperature

Detector wavelength: 320 nm

Injection volume: 20μl

[0056]

3. Calibration curves

[0057]

□ Preparation of standard stock solutions: The standard was prepared by weighing 4.95 mg, and dissolving and diluting with methanol in a 25 ml volumetric flask to obtain standard stock solutions for the calibration curves.

[0058]

□The Calibration Curves: From the stock solutions 0.4, 0.8, 1.2, 1.6, and 2.0 ml were weighed, dissolved, and diluted with methanol in 2 ml volumetric flask to obtain standard solutions at concentrations of 39.6 μg/ml, 79.2 μg/ml, 118.8 μg/ml, 158.4 μg/ml, and 198 μg/ml, respectively. A total of 20 μL of each standard solution was subject to HPLC quantitative analysis. A calibration curve was generated to

confirm the linear relationship between the peak area ratio (Y axis) and the concentrations of the standard (X axis) in the test samples. The calibration curves were found to be linear and could be described by the regression equations Y=20.139 X – 154.35, with coefficient R^2 = 0.9994. The range of calibration curves was 0.792 – 3.96 μg, and the retention time of epimeredinoside A was 9.55 min.

[0059]

4. Samples analysis

[0060]

Preparation of the standard solutions: The standard was accurately weighed, dissolved, and diluted with methanol in a volumetric flask to obtain standard solutions. A total of 20 μL of standard solution was subject to HPLC quantitative analysis and the peak area was recorded. The contents of epimeredinoside A was calculated using the calibration curves accordingly, see Fig 2.

[0061]

Preparation of the sample solutions: The extracts of *Epimeredi indica* root (176.66 mg) were accurately weighted, and extracted with by ultrasonication at room temperature for 2 times, then centrifuged. The supernatants were combined and diluted with water in a 10 ml volumetric flask. The solution was filtered through a syringe filter (0.45 μm).

[0062]

The sample solutions were subjected to HPLC analysis as described above, shown in Fig. 3

[0063]

The content of epimeredinoside A in samples were calculated according to the calibration curves.

[0064]

Peak area (Y): 383.380.

[0065]

The concentration X is 26.70 μg/ml according to the regression equations Y=20.139 X – 154.35.

[0066]

The content of epimeredinoside A in the sample was 0.15% by the equation X*10 / Sample Amount*100%.

[0067]

Example 3 - Preparation of a granulate

[0068]

Formula:

Extracts of *Epimeredi indica* Root	150 g
Lactose	50 g
Stearate Magnesium	2 g

[0069]

Methods: The extracts of *Epimeredi indica* Root which were prepared as described in Example 2 were mixed with lactose and stearate magnesium, and then sieved. The granulate was obtained by sieving again. The content of epimeredinoside A was 0.17 %.

[0070]

Example 4 - Preparation of a granulate

[0071]

Formula:

Extracts of the *Epimeredi indica* Root	130 g
Lactose	70 g
Stearate Magnesium	1 g

[0072]

Methods: The extracts of *Epimeredi indica* Root which were prepared as described in Example 2 were mixed with lactose and stearate magnesium, and then sieved. The granulate was obtained by sieving again. The content of epimeredinoside A was 0.13 %.

[0073]

Example 5 - Preparation of a Capsule

[0074]

Formula:

Extracts *Epimeredi indica* Root	110 g
Lactose	90 g
Stearate Magnesium	1 g

[0075]

Methods: The extracts of *Epimeredi indica* Root which were prepared as described in Example 2 were mixed with lactose and stearate magnesium, and then sieved. The grain was sieved again. The capsules were filled with the fine grain.

The content of epimeredinoside A was 0.27 %.

[0076]

Example 6 - Preparation of a Tablet

[0077]

Formula:

The extracts of *Epimeredi indica* Root	230 g
Cellulose, Microcrystalline	20 g
Carboxymethyl Starch, Sodium	3 g
Polyvinylpyrrolidone	1 g
Pulvis Talci	1 g
Stearate, Magnesium	1 g

[0078]

Methods: The Microcrystalline Cellulose, Sodium Carboxymethyl Starch and other materials were mixed in a mortar, and the extracts of *Epimeredi indica* Root which were prepared as described in Example 2 were added. The powder was shaped in a muller. The fine powder was granulated, dried and Magnesium Stearate added. The granulate was tableted and coated. The content of epimeredinoside A was 0.23 %.

[0079]

Example 7 - Preparation of a Tablet

[0080]

Formula:

The extracts of *Epimeredi indica* Root	300g
Cellulose, Microcrystalline	26g
Carboxymethyl Starch, Sodium	2.8g
Polyvinylpyrrolidone	2.8g
Pulvis Talci	2.8g
Stearate, Magnesium	1g

[0081]

Preparation was carried out according to the method mentioned in Example 6. The concentration of epimeredinoside A was 0.22%.